Unclassified
SECURITY CLASSIFICATION OF THIS PAGE

(2)

REPORT DOCUMENT

AD-A192 750

DTIC ELECTE MAR 15 1988 S D

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RE FILE COPY
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release Distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) AFOSR-TR- 88-0253
6a. NAME OF PERFORMING ORGANIZATION The Board of Trustees of the University of Illinois	6b. OFFICE SYMBOL (If applicable)
6c. ADDRESS (City, State, and ZIP Code) 506 S. Wright St. Urbana, IL 61801	7a. NAME OF MONITORING ORGANIZATION Air Force Office of Scientific Research 7b. ADDRESS (City, State, and ZIP Code) Building 410 Bolling Air Force Base, D.C. 20332
8a. NAME OF FUNDING/SPONSORING ORGANIZATION AFOSR	8b. OFFICE SYMBOL (If applicable) NL
8c. ADDRESS (City, State, and ZIP Code) Building 410 Bolling Air Force Base, D.C. 20332	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER AFOSR 84-0317 10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102F	2312	A5	

11. TITLE (Include Security Classification)

Structure and Function of Cytochrome P-450 Genes (unclassified)

12. PERSONAL AUTHOR(S) Byron Kemper

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 9/1/86 TO 8/31/87	1/25/88	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Cytochrome P-450, Regulation of drug metabolism, Gene structure, Transcription, Phenobarbital-induction Complementary Deoxyribonucleic Acid's

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The cytochrome P450IIC2 (previously designated P-450PBc) subfamily of the phenobarbital-inducible family of cytochrome P-450 contains several closely related members. We have reported previously the characterization of cDNA's for four of these P-450's, partial characterization of three of the corresponding genes and have shown that phenobarabital-induction can largely be accounted for by an increase in transcription rates. In year 3 we have continued our analysis of the genes of this subfamily concentrating on the 5' flanking regions which probably contain regulatory regions. To complement our previous characterization of the 3' end of the P450IIC3 gene we have isolated a lambda phage clone containing the 5' portion of the gene. This cloned genomic fragment contained exons 2-6 and combined with the earlier work defines the structure of exons 2-9 into the 3' flanking region spanning more than 25 kilobase pairs. We are continuing our search for a cloned genomic fragment containing exon 1 and the 5' flanking region. We have also characterized a genomic fragment containing exons 1-5 of the phenobarbital-in-

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Cockerham/Dr. T. Jan Cerveny	(202)767-5021	

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

29 JAN 1989

Atch 3

10 cont'd

ducible P450IIC4 gene. Mapping of the 5' end of the mRNA by the single strand nuclease technique indicated at least two start sites for RNA transription that correspond appropriately to two promoter TATA sequences. Significant similarities between P450IIC4 and another phenobarbital-inducible gene, P450IIC2, extend only about 150 base pairs into the 5' flanking region. These conserved regions presumably contain regulatory signals for expression of the genes. We are presently continuing the isolation and characterization of the genes and have begun to study the effect of phenobarbital on the chromatin structure of these genes and to characterize nuclear proteins that bind to the genes.

ANNUAL TECHNICAL REPORT

RESEARCH GRANT AFOSR 84-317

September 1, 1986 to August 30, 1987

Byron Kemper, P.I.

SUMMARY

The cytochrome P450IIC2 (previously designated P-450PBc) subfamily of the phenobarbital-inducible family of cytochrome P-450 contains several closely related members. We have reported previously the characterization of cDNA's for four of these P-450's, partial characterization of three of the corresponding genes and have shown that phenobarabital-induction can largely be accounted for by an increase in transcription rates. In year 3 we have continued our analysis of the genes of this subfamily concentrating on the 5' flanking regions which probably contain regulatory regions. To complement our previous characterization of the 3' end of the P450IIC3 gene we have isolated a lambda phage clone containing the 5' portion of the gene. This cloned genomic fragment contained exons 2-6 and combined with the earlier work defines the structure of exons 2-9 into the 3' flanking region spanning more than 25 kilobase pairs. We are continuing our search for a cloned genomic fragment containing exon 1 and the 5' flanking region. We have also characterized a genomic fragment containing exons 1-5 of the phenobarbital-inducible P450IIC4 gene. Mapping of the 5' end of the mRNA by the single strand nuclease technique indicated at least two start sites for RNA transription that correspond appropriately to two promoter TATA sequences. Significant similarities between P450IIC4 and another phenobarbital-inducible gene, P450IIC2, extend only about 150 base pairs into the 5' flanking region. These conserved regions presumably contain regulatory signals for expression of the genes. We are presently continuing the isolation and characterization of the genes and have begun to study the effect of phenobarbital on the chromatin structure of these genes and to characterize nuclear proteins that bind to the genes.

A. RESEARCH OBJECTIVES

The overall goal and the specific aims of this project remain the same as described in the initial application. The overall goal is to understand the regulated biosynthesis of rabbit liver cytochrome P-450. To achieve this goal, we are studying the structure of the genes for these enzymes, the mechanisms by which phenobarbital induces the activity of the enzymes and the nature of the mRNA's. Listed below are the specific objectives for the third year (1986-1987) that were in the original application. Our principle efforts during the past year have been to isolate and characterize the remaining genes in the P450IIC2 subfamily. Objective 5 has been eliminated because the P450IIC2 subfamily genes are sufficient for our studies on phenobarbital induction.

1. Examine whether the P-450PBc cDNAs code for actual P-450's.

2. Isolate and characterize cloned DNA fragments containing the P-450PBc genes.

3. Evaluate the evolutionary relationships and possible functional significance of structure by comparative analysis of amino acid and nucleotide sequences of the P-450PBc proteins with each other and other P-450's.

4. Analyze the regulation of the transcription of the P-450PBc genes introduced into mammalian cells.

5. Isolate cloned cDNAs of other rabbit liver P-450s that cannot be detected by hybridization to previously cloned cDNAs.

6. Determine the role of synthesis and degradation in the induction of P-450PBc mRNA.

B. STATUS OF RESEARCH:

The status of the research for each of the specific aims listed on the previous page is described below.

1. Examine whether the P-450PBc cDNAs code for actual P-450's. As noted previously, proteins corresponding to P450IIC2 and P450IIC3 have been identified. The characterization of the genomic clone for P450IIC3 below allowed us to compare additional derived protein sequence with that determined for the protein, P4503b, which corresponds to P450IIC3. The first figure shows the differences between the sequences derived from the gene, cDNA and protein. Several differences are noted that may result from polymorphisms or from errors in sequencing. The close correspondence between the gene and cDNA sequence suggest that several misassignments of the protein sequence were made.

2. Isolate and characterize cloned DNA fragments containing the P-450PBc genes. A second lambda phage clone coresponding to the P450IIC3 gene was isolated and characterized by DNA sequencing of the exon regions and by restriction mapping to determine distances between the exons. The new clone contained sequences corresponding to exons 2 through 6, however, it did not overlap with the first fragment that had been characterized so that the distance between exon 6 and 7 is not yet known. This gene spans at least 25 kbp (figure 2). The gene sequence differed from the cDNA sequence in only 2 of 1400 bases and predicted 7 differences of 435 amino acids in the sequence from the protein. In a similar fashion we have characterized a cloned DNA fragment that corresponds to P450IIC4. This fragment of 15 Kbp contains the 5' flanking region, and the first 5 exons of the gene (figure 3). There is evidence that there are at least three genes very closely related to P450IIC4, one of which is P450IIC5. Of the 862 exonic bases in the gene sequence there were only 3 differences with the reported P450IIC4 cDNA and 42 with the P450IIC5 sequence providing reasonable assurance that the gene corresponds to P450IIC4. Previous work with the 3' untranslated region of a P450IIC4 cDNA had indicated that this gene was inducible with phenobarbital. To show that the 5' P450IIC4 gene sequence was also inducible by phenobarbital, oligonucleotides were synthesized that were specific for either P450IIC5 or P450IIC4. Hybridization of these probes to mRNA from control and phenobarbital-treated livers indicated that the C5 mRNA was not induced and the C4 mRNA was induced by several-fold as expected. We have also mapped the 5' start site of transcription by protection from single-strand specific nuclease of DNA fragments hybridized to mRNA. As shown in figure 4, a major protected fragment started at an A residue indicated as position 1 and a second minor start site was indicated by a series of bands at about 54 bp prior to position 1. For each of these two start sites, as shown by the sequence above the figure, a TATA-like sequence is present in the appropriate position to direct the initiation of RNA transcription.

3. Evaluate the evolutionary relationships and possible functional significance of structure by comparative analysis of amino acid and nucleotide sequences of the P-450PBc proteins with each other and other P-450's. We have compared the sequence of P450IIC4 with the sequence of P450IIC2 by dot matrix analysis as shown in figure 5. In the 5' flanking region there is considerable similarity around the TATA sequence at -20 and three other regions of similarity in the -50 to -150 region. Little homology is noted beyond -150

suggesting that the primariy region conserved, presumably because of regulatory function, is in the -1 to -150 region. Small regions of homology not detectable by the dot matrix technique are also present, including sequences that have been shown to be liver-specific sequences in other genes. Establishing the significance of these regions will require functional studies of the transcription of the gene. A similar dot matrix analysis comparing either rabbit P450IIC2 or P450IIC4 with rat P450IIB1, another phenobarbital-inducible form, did not detect any similarity (figure 6).

4. Analyze the regulation of the transcription of the P-450PBc genes introduced into mammalian cells. As noted last year a P450-CAT gene was not expressed in a rat hepatoma line. We are taking a modified approach by introducing 5' flanking regions from P450 genes upstream of a CAT gene driven by the thymidine kinase promoter which is a weak promoter. This type of construction should allow us to define regulatory regions in the P450 genes.

5. Isolate cloned cDNAs of other rabbit liver P-450s that cannot be detected by hybridization to previously cloned cDNAs. No longer an objective.

6. Determine the role of synthesis and degradation in the induction of P-450PBc mRNA. As noted last year, the transcription studies strongly suggest that the major phenobarbital effects are on synthesis of mRNA. No additional studies have been done.

FIGURE LEGENDS

Figure 1. Differences between the protein sequence of P450IIC3 as derived from the gene, cDNA and protein. At positions of differences, the amino acids are shown. The amino acids at the analogous postion in P450IIC1, P450IIC2, P450IIC4 and P450IIC5 are also shown.

Figure 2. Schematic diagram of the gene structure of P450IIC3. Exons are indicated by the boxes; the position of open boxes have not been precisely determined within the R1 fragments. The corresponding DNA inserts of lambda phage clones are shown below the structure and the number of mismatches of the sequence with the cDNA and the derived protein with P450(3b) are also shown.

Figure 3. Schematic diagram of the gene structure of P450IIC4-like genes. Exons are indicated by the boxes and the two lambda phage clones that were characterized are indicated. The number of differences in the sequences with the cDNA sequences of P450IIC4 and P450IIC5 are also shown.

Figure 4. Mapping of the transcription start site of P450IIC4. Liver RNA from control or phenobarbital-treated rabbits was hybridized to a DNA fragment which was labeled on one end in the Exon 1 region and which extended into the 5' flanking region. After the hybridization, the sample was treated with mung bean nuclease (100 to 400 U) to digest unhybridized nucleic acids. The size of the protected DNA fragments were determined by electrophoretic analysis on a DNA sequencing gel and as markers the DNA fragment cleaved in a base-specific (sequencing) manner was also analyzed. The position of the major protected fragment is designated 1 and a second start site is indicated at -54 by

the protected fragments in this region. The sequence of the P450IIC4 gene in these regions is shown above the gel and the TATA sequences are underlined.

Figure 5. Comparison of sequences for P450IIC4 and P450IIC2 by dot matrix. In this analysis a point was plotted whenever 8 nucleotides in a window of 10 were identical in the two sequences. The positions of the initiator codon (ATG) and the TATA sequence are shown.

Figure 6. Comparison of sequences for P450IIC4 and P450IIB1 by dot matrix. The analysis was as described in Figure 5 except that points were plotted whenever 7 of 10 matches occurred.

PUBLICATIONS

1. Zhao, J, Leighton, J. K. and Kemper, B. (1987) Characterization of rabbit cytochrome P450IIC4 cDNA and induction by phenobarbital of related hepatic mRNA levels. Biochem. Biophys. Res. Commun. 146: 224-231.

2. Kemper, B., Bell, P., Chan, G., Govind, S. and Zhao, J. (1988) Structure of rabbit cytochrome P450IIC genes and regulation by phenobarbital. In Microsomes and Drug Oxidations, Taylor and Francis, London, in press.

3. Hankinson, O. and Kemper, B. (1987) Meeting report. Cytochrome P450 gene regulation. DNA 6: 515-517

4. Zhao, J. and Kemper, B. Comparison of the gene structure of two phenobarbital-inducible genes in the P450IIC subfamily. in preparation.

Personnel

Byron Kemper, Principal Investigator
Ging Chan, Graduate Research Assistant
Jian Zhao, Graduate Research Assistant
Maria Kyroudis, Visiting Research Specialist

Abstracts/presentations

Invitations to National and International meetings:

1. The Second International Workshop on P450 Gene Regulation at Airlie, Virginia sponsored by the NIH, April, 1987. Paper presented.

2. The Seventh International Symposium on Microsomes and Drug Oxidations at Adelaide, Australia, August, 1987. Paper presented.

Seminar invitations:

1. Department of Molecular and Cellular Biology, University of Oregon, April 1987.

8. INVENTIONS None

P450IIC3 AMINO ACID DIFFERENCES

POSITION	PROTEIN	cDNA	GENE	OTHER IIC PROTEINS
82	GLY	-	THR	ALA(4)
84	ILE	LYS	LYS	LYS(4)
90	TYR	ASP	ASP	ASP(4)
223	GLY	GAP	GAP	LEU(2) PHE(1) ILE(1)
257	SER	SER	LEU	VAL(2) ILE(2)
339	SER	MET	MET	MET(4)
343	SER	THR	THR	SER(4)
430	THR	ALA	ALA	ALA(2) THR(2)

Figure 1

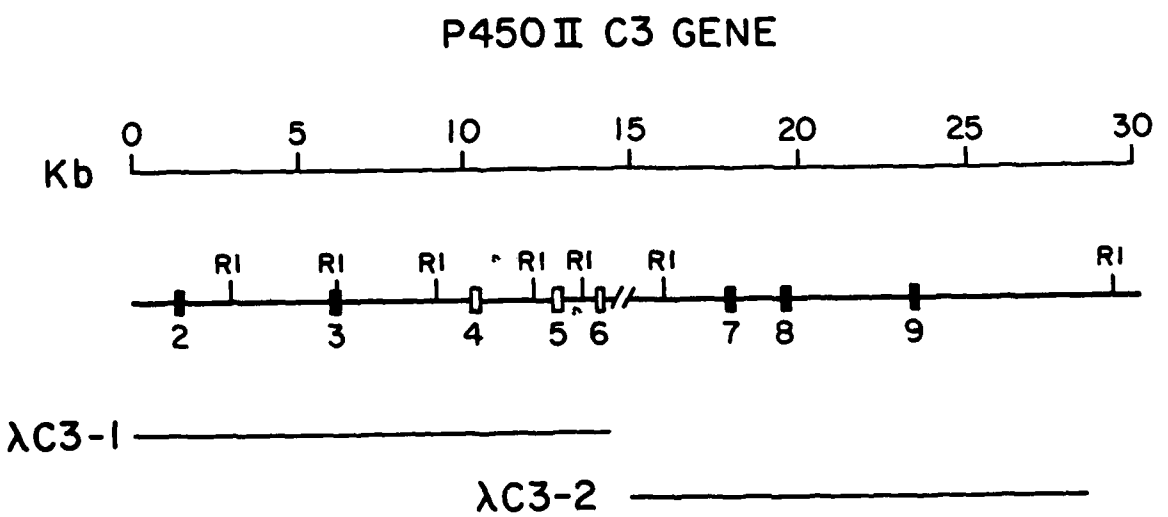

Figure 2

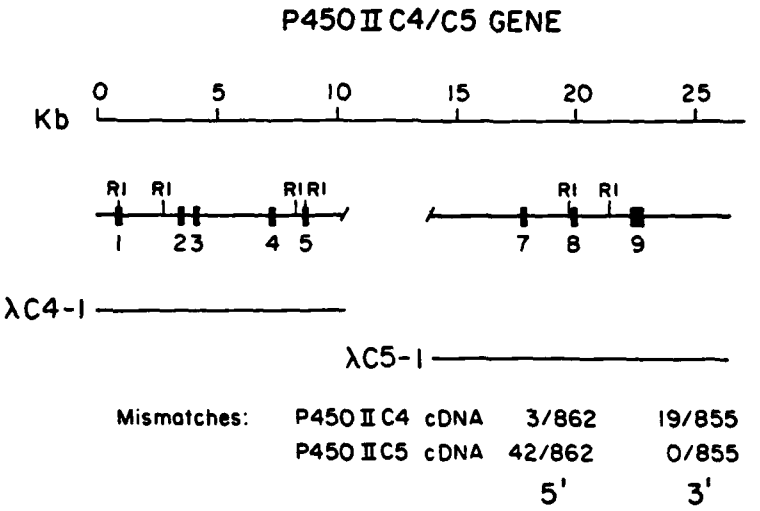

Figure 3

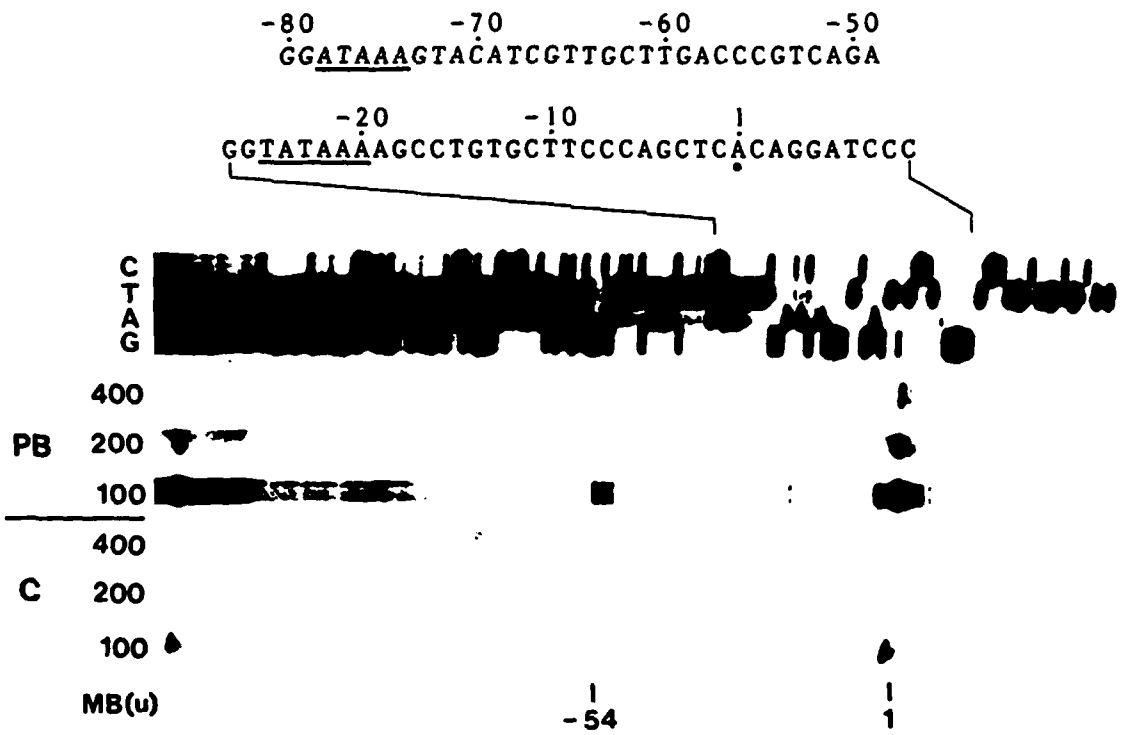

Figure 4

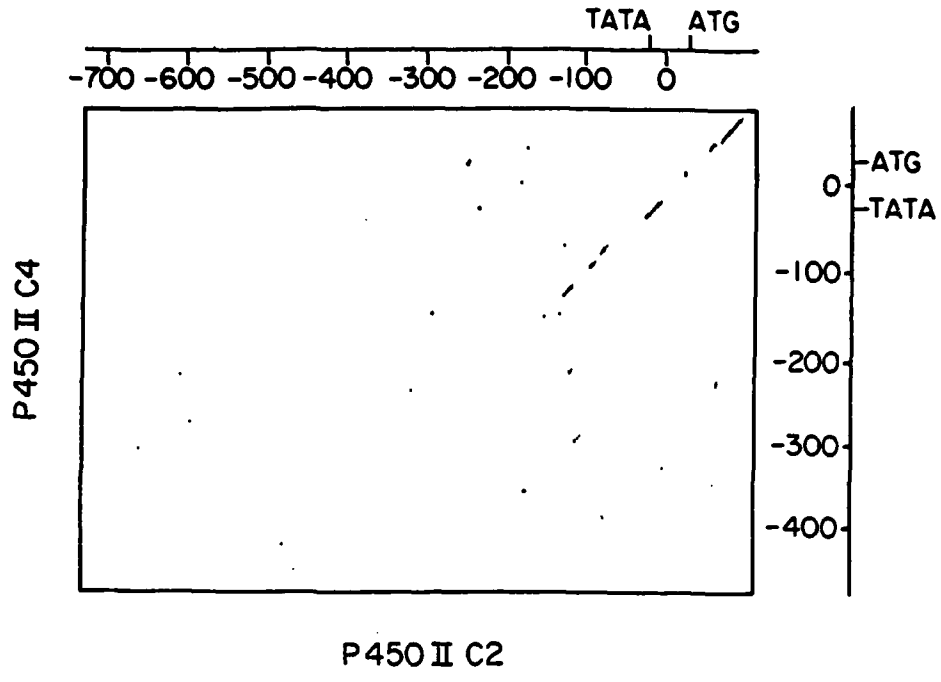

Figure 5

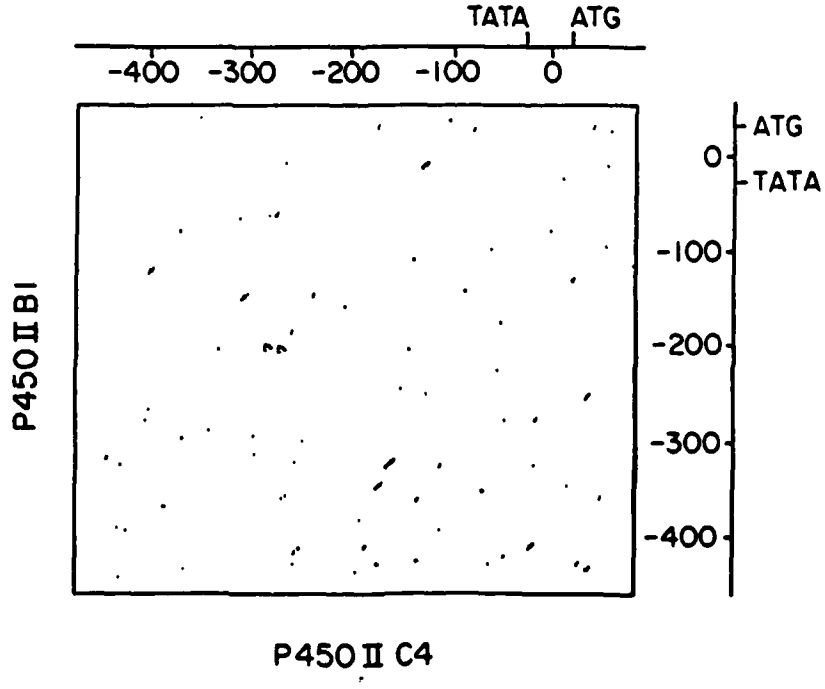

Figure 6